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# Screening of organic solvents for bioprocesses using aqueous-organic two-phase systems

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## Abstract

The application of conventional organic solvents is essential in several steps of bioprocesses in order to achieve sufficient economic efficiency. The use of organic solvents is frequently used either to partly or fully replace water in the reaction medium or as a process aid for downstream separation.

Nowadays, manufacturers are increasingly requested to avoid and substitute solvents with hazardous potential. Therefore, the solvent selection must account for potential environmental hazards, health and safety problems, in addition to fulfilling the ideal characteristics for application in a process.

For the first time, criteria including Environment, Health and Safety (EHS), as well as the technical requirements for reaction and separation have been reviewed, collected and integrated in a single organic solvent screening strategy to be used as a guideline for narrowing down the list of solvents to test experimentally. Additionally, we have also included a solvent selection guide based on the methodology developed in the Innovative Medicines Initiative CHEM21 (IMI CHEM21) project and applied specifically to water-immiscible solvents commonly used in bioprocesses.

**Keywords:** Organic solvents screening, Bioprocesses, Biphasic systems, Downstream processing, *In situ* product removal

## 1. Introduction

There is currently significant interest in the application of biotechnology to chemical manufacture, driven in part by the need to replace (or at least minimize) existing fossil feedstocks by renewable and sustainable ones. Likewise the chemical industry, and perhaps even more importantly the pharmaceutical industry, needs to use ever cleaner processes, with reduced reagent use and waste generation. For example, while the *E* factor is a measure of the amount of waste produced in a

process ( $E$  factor = kg waste / kg product) (Sheldon, 2017), it is perhaps more useful to examine the composition of the waste from a given process. This quickly motivates the need to reduce or replace the use of organic solvents, applied primarily for product recovery and purification. For this reason several pharmaceutical companies, academic groups and organisations like the ACS Green Chemistry Institute (GCI) Pharmaceutical Roundtable have successfully driven an agenda of solvent reduction and replacement (Constable *et al.*, 2007; Jessop *et al.*, 2015; Tucker and Faul, 2016). To a large extent this has been focused on chemical synthetic strategies. However, while this serves as a very valuable guidance for today, the range of industrial processes is changing. For example, already today several hundred small-molecule pharmaceutical production processes use one or more bioprocess steps (Buchholz *et al.*, 2012; Meyer *et al.*, 2013; Woodley, 2017). Indeed as industrial interest in cleaner synthesis grows it becomes clear that in the future many more bioprocesses will be implemented in industry (Cue and Zhang, 2009; Sheldon and Woodley, 2017). Even if fermentation and biocatalysis were to replace a significant fraction of the synthetic reactions in the fine chemical and pharmaceutical industry, it remains the case that the products still need to be recovered and purified. The downstream separation can include many potential unit operations which are dependent upon the product (as well as by-product and substrate) properties. Nevertheless, for most biocatalytic reactions and fermentations the product is often toxic (leading to an irreversible loss of activity) or inhibitory (leading to a reversible loss of activity) to the biocatalyst/microorganism at concentrations much lower than are the minimum required to feed a conventional downstream process. This has been the major motivation behind the implementation of *in situ* product removal (ISPR), where inhibitory or toxic products are removed during the reaction (either at the site of the reaction, or else in a recycle loop) (Van Hecke *et al.*, 2014; Woodley *et al.*, 2008; Zou, 2014). Various methods have been proposed including the use of adsorption, pervaporation, perstraction, and crystallization. Extensive reviews have been written on this topic and a number of industrial processes use the technology (Carstensen *et al.*, 2012; Dafoe and Daugulis, 2014; Freeman *et al.*, 1993; Lye and Woodley, 1999; Stark and von Stockar, 2003; Van Hecke *et al.*, 2014; Woodley *et al.*, 2008). Of particular interest is that polymers have been used in many ISPR solutions (Phillips *et al.*, 2013) and can potentially be an effective, safer and cheaper alternative to the use of organic solvents (Dafoe and Daugulis, 2014). Regardless of the type of phase used to recover product it is clear that systematic selection methods are required. On this premise we recognized that one of the most used separation methods (aqueous-organic liquid-liquid extraction) could in particular benefit from a more systematic screening procedure for the organic solvent. In this review, for the first time, the criteria to screen for solvents for a bioprocess are integrated in a single report, accounting for both the technical, as well as EHS requirements which as we have indicated earlier are a prerequisite for industrial implementation. The collection of these criteria forms the basis of a screening procedure in particular focused on biphasic systems in bioprocesses in order to narrow down the number of solvents to be tested experimentally. In this paper in contrast to previous publications (Elgue *et al.*, 2006; Gani, 2006; Zhou *et al.*, 2014), we deliberately restrict ourselves to bioprocesses using enzymes or microorganisms, to manufacture chemical products. We consider this screening procedure essential for the scientific community involved in the early stage development and research of new bioprocesses. Interestingly, this rationale is supported by journals such as ChemSusChem (Kemeling, 2012) which has specifically asked authors to justify their choice of solvents in submitted manuscripts and if possible to consider replacing harmful ones.

## 2. Use of organic solvents in bioprocesses

Whilst the use of water-miscible organic solvents (e.g. ethanol, dimethyl sulfoxide) to help solubilize poorly-water soluble organic compounds in single phase biocatalytic systems has been

widely reported in the scientific literature, such systems may give only a 10-20% increase in substrate and/or product concentration (Sheldon and Pereira, 2017). Additionally, with only a few exceptions, such polar solvents strip essential water from the biocatalyst resulting in a loss of enzyme stability (Gorman and Dordick, 1992; Kamal *et al.*, 2013; Taher and Al-Zuhair, 2017; Yang *et al.*, 2004). On the other hand, essentially water-immiscible organic solvents (containing only small amounts of water, at concentrations less than saturation) like n-hexane, t-butyl methyl ether etc. can be used for lipase reactions run in a synthetic direction (to avoid hydrolysis) (Bose and Keharia, 2013; Carvalho *et al.*, 2015; Devi *et al.*, 2017). In this paper we will focus on the third case, where water-immiscible solvents are used in a distinct phase from the aqueous phase, to form a two-liquid phase system.

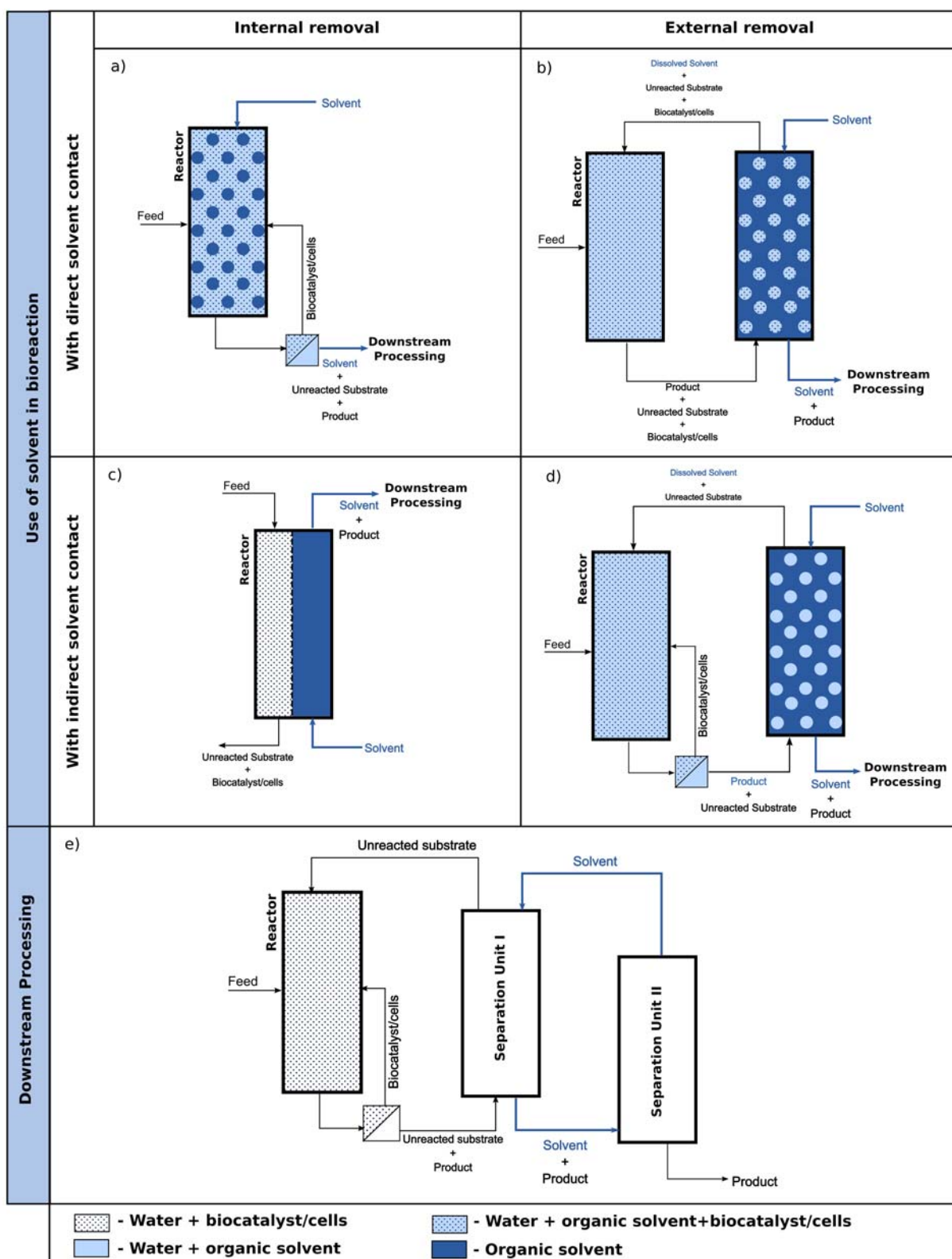
Here the organic solvents are used for substrate supply, or product removal, in order to overcome the low water-solubility of organic compounds and enzyme inhibition by substrate or product. Potentially, the solvent may also be used to overcome an unfavourable equilibrium, although this requires sufficient driving force to be effective. In this way, the application of two-liquid phase systems improves the bioreaction space-time yield (productivity) as well as the product concentration fed to the downstream process, and in some cases the selectivity (Boghigian *et al.*, 2011; Dafoe and Daugulis, 2014; Jung *et al.*, 2013; Mutti and Kroutil, 2012).

## 2.1 Bioreaction systems

Several considerations are important in aqueous-organic two-phase biocatalytic systems. The organic phase may be deleterious to the biocatalyst in two ways; either by the presence of the interface (Martínez-Aragón *et al.*, 2009; Perez-Rodriguez *et al.*, 2003) or by the amount of organic solvent dissolved in the aqueous phase which may cause biocatalyst inactivation (Bes *et al.*, 1995; Stepankova *et al.*, 2013). Both appear to be important, but in many cases the biocatalyst needs to be kept away from the interface.

Despite the downside described above the introduction of an organic solvent in the bioreaction system presents several advantages such as the dissolution of substrates and products at higher concentrations in the reactor than would otherwise be achievable. This means that the downstream process can be fed at high concentrations, while avoiding inhibitory concentrations of substrate or product in the aqueous reaction environment (Hua and Xu, 2011; Lima-Ramos *et al.*, 2014). Easier product recovery may also result from the fact that the solvent has a low boiling point, facilitating evaporation (Dafoe and Daugulis, 2014). Likewise when designing an *in-situ* product removal (ISPR) process, the mode of contact (direct or indirect) between the biocatalyst and the organic phase which removes the product, should be considered (Stark and von Stockar, 2003; Woodley *et al.*, 2008). A bioreaction system with direct solvent contact can be characterized by the direct exposure of the biocatalyst/cells to the organic solvent [Figure 1 a) and b)]. For a bioreaction system with indirect solvent contact [Figure 1 c) and d)] the biocatalyst is not in contact with the organic solvent (Stark and von Stockar, 2003; Woodley *et al.*, 2008).

In Figure 1, two possibilities for running systems with direct contact are presented: a) corresponds to the exposure of the biocatalyst to organic solvent within the reactor and b) corresponds to the direct contact in a different vessel to the reactor through an external loop. Configuration a) has the advantage that both reaction and product removal take place in the same vessel and therefore the equipment costs are lower. Configuration b) reduces the contact time between the biocatalyst and the organic solvent by introducing an external loop through a separation unit. However, the choice



**Figure 1** – Three process configurations for a bioreaction in an aqueous-organic two-phase system. Figure adapted from Stark and von Stockar, 2003.

of solvent has to ensure that the solvent does not deactivate the biocatalyst/microorganism and the product has a high enough affinity and solubility.

Additionally, two configurations for indirect contact are presented in Figure 1: c) corresponds to a biphasic reactor with a membrane which separates the two liquid phases and d) corresponds to the separation of the biocatalyst/cells from the reactor medium and use of another vessel for the product removal. In systems such as c) there is usually a physical barrier such as a membrane which prevents the contact of the biocatalyst with the solvent (Stark and von Stockar, 2003; Woodley *et al.*, 2008). In the configuration d), the biocatalyst/microorganism is never in direct contact with the solvent. The biocatalyst/microorganism is separated from the product and is recycled to the reactor. The medium with product dissolved, in its turn, enters a liquid-liquid extraction unit where the product is partitioned to the organic solvent and the medium that exits the vessel is recycled to the reactor.

The choice of solvent for a two liquid-phase system with direct contact is more difficult than for an indirect contact configuration since it must be compatible with the biocatalyst/microorganism and therefore requires a careful study of its toxic effects.

## 2.2 Downstream processing

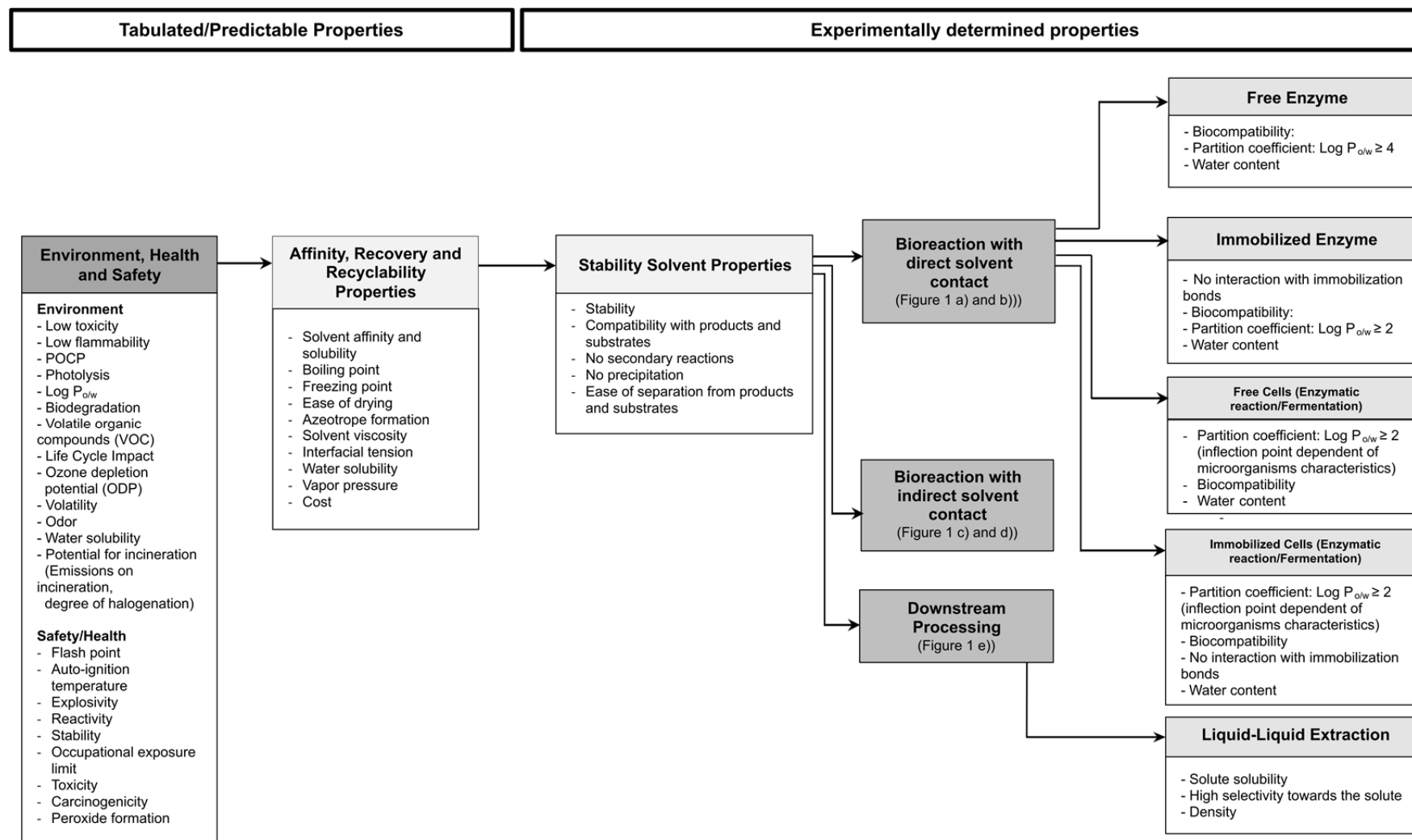
Organic solvents play an important role as separation and purification agents for small-molecule chemical products from bioprocesses since they allow easy recovery of organic compounds. The use of water as a solvent may present some challenges for downstream processing such as separation difficulties, and its high specific heat capacity implies high energy consumption in distillation and difficulties rapidly heating and cooling (Adams *et al.*, 2003). Moreover, the solubility of many of the most interesting compounds is often very low in water which implies excessive amounts of water in order to recover small amounts of product, resulting in high costs. When choosing an organic solvent, it should be possible to separate it from the aqueous phase as well as recover the desired products from the solvent as shown in Figure 1 e) (Gu, 2000; Koch, 2015). This should also enable options for recycling the solvent if viable, which could help optimize the economic feasibility of a given process, due to lower overall solvent use. Nowadays, the recycling of solvents is a common practice in industry. Besides the advantages mentioned above, the separation costs for isolating a product from an organic solvent can be much lower when compared to an aqueous system.

The determination of the exact downstream processing conditions depends not only on the nature of the product (solid or liquid) but also on the phase in which the product is primarily soluble. For a two-liquid phase system (i.e. with two immiscible phases), the operation unit mostly used to purify products is liquid-liquid extraction. Concerning energy consumption, liquid-liquid extraction can be more attractive since it is a less energy consuming process compared to distillation and gives a relatively high efficiency for product recovery (Kurzrock and Weuster-Botz, 2010; Stratakos and Koidis, 2016).

## **3. Overview of criteria to screen solvents for an industrial bioprocess**

The list of solvents applicable to industrial processes is extensive and thus, the choice of the optimum solvent can be a significant challenge. Hence, at an early stage of process development, it is necessary to make a screening of solvents for evaluation of their suitability for the industrial process.

Figure 2 shows a screening procedure which is divided in four evaluation categories: (1) environment, health and safety, (2) affinity, recovery and recyclability properties, (3) stability and (4)



**Figure 2** – Overview of criteria for choosing an organic solvent for a bioprocess divided in tabulated properties in scientific literature and experimentally determined properties which are dependent on system and compounds characteristics.

1 application. The screening is also divided between tabulated properties which are already available  
2 in the literature and experimentally determined properties, which are dependent on the  
3 characteristics of the system and have to be experimentally investigated in order to evaluate the its  
4 performance.

5 The purpose of the screening procedure is to help narrowing the list of possible solvents to be  
6 applied in a bioprocess by evaluating the most important criteria first and eliminating those solvents  
7 which do not fulfill the requirements. The methodology starts by evaluating solvents in terms of  
8 environment, health and safety issues because this is the greatest concern for process development.  
9 Indeed, in order to implement a process it is necessary to fulfill legal and regulatory requirements in  
10 this category. Subsequently, solvents are evaluated in terms of recovery and recyclability properties  
11 and finally the list is shortened by considering those which fulfill the criteria for application in a  
12 given bioprocess.

13 Ultimately an experimental investigation has to be performed since the solvent is selected according  
14 to the specific system under study. Nevertheless, some of the listed properties such as Log  $P_{o/w}$   
15 provide a direction for the search.

### 16 17 3.1 Criteria to screen for organic solvents with low hazard environmental, health and safety (EHS) 18 issues

19 The adequate selection of solvents is dependent on their suitability for a given application.  
20 However, considerations regarding solvent recovery, solvent release as well as safety at an  
21 industrial site have particular importance. Hence, the primary category to assess is their impact on  
22 environmental, safety and health. It is necessary to take several parameters into account such as  
23 those quantifying the environmental impact (ecotoxicity, flammability, ozone depletion,  
24 incineration potential, etc). Regarding health and safety, some of the parameters are: toxicity &  
25 occupational exposure, auto-ignition temperature, boiling point, flash point, explosivity, reactivity  
26 and vapor pressure; these are particularly important considerations where a bioprocess is run in the  
27 presence of air or oxygen. Solvent selection guides are available, and some institutions and  
28 companies have also made studies to evaluate the hazards of the solvents and suggested alternative  
29 solvents which could substitute the most hazardous ones (Alfonsi *et al.*, 2008; American Chemical  
30 Society (ACS), 2011; Elgue *et al.*, 2006; Henderson *et al.*, 2011; Prat *et al.*, 2016, 2013).

### 31 32 3.2 Criteria to evaluate the recovery strategies and affinity and stability of an organic solvent

33 When screening for organic solvents for a particular application in a process there are initially  
34 several considerations to take into account including the affinity, stability and recovery of the  
35 solvent.

36 The affinity of a given solvent towards a solute is a fundamental property to consider when  
37 choosing a solvent since it determines the viability of the solvent application. Even though this  
38 property is very specific for the process, it is possible to find data bases with information for  
39 specific solute-extractant pairs such as, (Dortmund Data Bank, 2018). In those cases where the  
40 information is not tabulated, the ternary phase behavior can be predicted using thermodynamic  
41 methods such as NRTL, UNIFAC and UNIFAQ. The successful application of these predictable  
42 methods has been widely reported in scientific literature (Abildskov *et al.*, 2001; Brennan *et al.*,  
43 2012; Bruce and Daugulis, 1991; Cheng and Wang, 2010, 2007; Domańska *et al.*, 2015; Ellegaard  
44 *et al.*, 2009; Janseen *et al.*, 1993; Malinowski, 2001, 1999; Malinowski and Daugulis, 1994;  
45 Modarresi *et al.*, 2008; Priebe and Daugulis, 2018; Scilipoti *et al.*, 2014). The reader should also  
46 note that any solvent selected in this way will still need be experimentally tested, not only for  
47 affinity but also for emulsion formation and biocompatibility.



When choosing a solvent for a bioprocess it is also necessary to take into consideration parameters such as viscosity, vapor pressure and melting point (Martínez-Aragón *et al.*, 2009; Tzia and Liadakis, 2003). The values of all these parameters should be low enough to ensure ease of handling and storage. For example, highly viscous solvents lead to problems effective liquid-liquid mass transfer. With respect to recovery and recyclability, the boiling point is an important parameter to consider, especially if the separation is done by distillation (Barwick, 1997). There are several other criteria to take into consideration as well, such as the ease of drying and azeotrope formation (Smallwood, 1996; Tzia and Liadakis, 2003). It is relevant to consider that all the factors mentioned above are very important in order to run a process with a solvent and solvent selection can be a delicate balance between the different parameters. The properties above are already tabulated and can be used for screening solvents and reduce the number of solvents to be tested. The non-precipitation, non-reactivity and chemical stability in the reaction system of the solvent are also important factors to consider (Tzia and Liadakis, 2003). Likewise the solvent should be stable and not interact with the reaction solutes (e.g. substrate(s) and product(s)) and cause secondary reactions. Needless to say, being able to operate the process safely is of paramount importance. Since most of these properties are dependent on the characteristics of an individual system, experimental work is necessary in order to assess the suitability of the solvent for the process. Therefore, these criteria should be evaluated in the end of the screening process to a very short list of solvents already chosen considering the tabulated properties.

### 3.3 Criteria for screening organic solvents as part of a reaction medium in two-liquid phase systems with free or immobilized biocatalyst/microorganisms

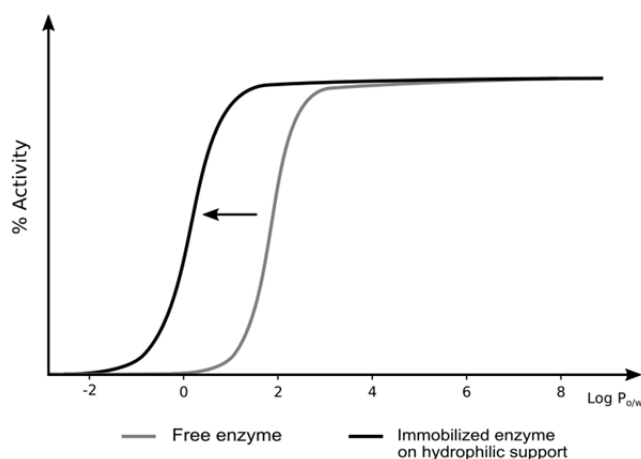
There are some specific challenges related to the use of solvents in bioreactions. As mentioned earlier, solvents can be damaging to the biocatalyst, causing degradation and inactivation. For an enzymatic reaction in a two-liquid phase system, there are some basic principles that can be followed in order to shorten the list of feasible solvent candidates for initial testing. The solvent should be as apolar as possible. Nevertheless, it should be noted that for such systems the aqueous-organic interface can also have toxic effects on the biocatalyst. The Log  $P_{o/w}$  value is the accepted parameter for defining the polarity of a solvent. Hence, Log  $P_{o/w}$  is useful for describing the influence of a solvent on enzyme activity. In the scientific literature, high partition coefficients (Log  $P_{o/w} > 4$ ) are considered suitable, whilst those with lower values have frequently been found toxic to biocatalysts (Halling, 1994; Laane *et al.*, 1987; Straathof, 2003).

Solvents with Log  $P_{o/w}$  values higher than 4 present a low solubility in water and, practically, the enzyme dissolved in the aqueous phase does not have contact with the solvent and is able to support effective product synthesis. On the other hand polar solvents with low Log  $P_{o/w}$  values (Log  $P_{o/w} < 2$ ) are more soluble in water and consequently remove the essential water from the enzyme and disrupt its conformation with attendant deactivation (Soo *et al.*, 2003). Several authors have reported the effect of solvents on the performance of enzymes and have shown that enzymes present better activity in media containing solvents with high Log  $P_{o/w}$  values (Bemquerer *et al.*, 1994; Koutinas *et al.*, 2018; Lara and Park, 2004; Valivety *et al.*, 1991; Zaks and Klivanov, 1985).

Interestingly, whilst the partition coefficient (Log  $P_{o/w}$ ) is an important parameter to assess the suitability for an organic solvent for soluble enzymes, it has also been found useful for immobilized enzyme systems, although with a more relaxed requirements. For example it has been possible to achieve good enzyme performance in biphasic systems using immiscible organic solvents with lower Log  $P_{o/w}$  values (range 1-3) (Chaplin *et al.*, 2001; Reslow *et al.*, 1987).

This indicates that the immobilization of the enzyme results in a shift of the Log  $P_{o/w}$ -activity curve as shown in Figure 3 (Laane *et al.*, 1986; Mionetto *et al.*, 1994). Consequently, with immobilized

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100

**Figure 3**-Schematic representation of enzymatic activity for both free enzyme and immobilized enzyme on a hydrophilic support plotted against  $\text{Log } P_{o/w}$  of the solvent. Figure adapted from Mionetto *et al.* 1994.

101 enzymes there is a broadening of the solvent polarity range and an increased number of suitable  
102 solvent options. In these reaction systems, it is believed that the support retains the water molecules  
103 and therefore stabilizes a water layer around the enzyme molecules. The water layer protects the  
104 enzyme molecules and therefore makes them more stable even in organic solvents with lower  
105 partition coefficients.

106 From the different studies reported, we can conclude that  $\text{Log } P_{o/w}$  should only be used as a  
107 guideline for screening biocatalyst-compatible solvents. In fact, it is not possible to determine the  
108 suitability of the solvent without performing experiments. Some exceptions to the guideline have  
109 been reported (Cantarella *et al.*, 1993; Geok *et al.*, 2003; Gonçalves *et al.*, 1997).

110 Furthermore, the characterization of enzyme performance in organic media has often been reported  
111 in an inconsistent manner. So while some authors report the enzyme activity (Mionetto *et al.*, 1994),  
112 or specific activity (Norin *et al.*, 1988), other report the residual activity (Geok *et al.*, 2003; Reslow  
113 *et al.*, 1987) and others again, the reaction conversion or yield (Chaplin *et al.*, 2001; Koutinas *et al.*,  
114 2018; Lara and Park, 2004). These inconsistencies mean that drawing conclusions about the use of  
115  $\text{Log } P_{o/w}$  as a parameter for solvent selection is difficult.

116 For whole-cell biocatalytic systems and fermentation, the relation between cellular activity of  
117 different microorganisms against  $\text{Log } P_{o/w}$  is also represented by a sigmoidal curve, similar to that  
118 for soluble enzyme (Laane *et al.*, 1987). Several authors (Bassetti and Tramper, 1994; Cruz *et al.*,  
119 2001; Fragnelli *et al.*, 2012; Neumann *et al.*, 2005; Rojas *et al.*, 2004; Silva *et al.*, 2010) have  
120 studied the relationship between cellular activity and  $\text{Log } P_{o/w}$ . From their results, unsurprisingly it  
121 is possible to conclude that the inflection points between toxic and non-toxic solvents vary  
122 significantly between different microorganisms. Bruce and Daugulis have proposed that the  
123 tolerance of the microorganism is dependent on the characteristics of the cellular membrane (L J  
124 Bruce and Daugulis, 1991). Whole-cells biocatalysis using organic media has been reviewed and  
125 the authors concluded that the inflection point of the sigmoidal curve is in general above the value  
126  $\text{Log } P_{o/w} 2$  (Heipieper *et al.*, 2007; León *et al.*, 1998). Nonetheless, it is still necessary to perform  
127 experimental screening work for the microorganism of interest. Some authors have engineered the  
128 microorganisms, in order to improve microorganisms tolerance to solvents, and in this way have

129 adapted a specific cell to have tolerance to a specific solvent (Mukhopadhyay, 2015; Volmer *et al.*,  
130 2014; Zhang *et al.*, 2015).

131 Regarding bioreactions in water-organic solvent two-phase systems with immobilized enzymes or  
132 cells, the toxicity of the solvent to the biocatalyst/microorganism is a crucial factor to consider  
133 when screening for organic solvents. However, the interaction of the solvents with the  
134 immobilization matrix is also an important factor to take into consideration. Immobilization by  
135 adsorption is the simplest method and is characterized by reversible surface interactions between  
136 enzyme/cells and the support material. The interaction forces can be van der Waals forces, ionic and  
137 hydrogen bonding interactions. Since these forces are weak, desorption can occur in the presence of  
138 organic solvents (Brena *et al.*, 2013; Dwevedi, 2016). Synthetic polymer resins can be prone to  
139 swell with certain classes of solvent. On the other hand, porous silica and porous glass have been  
140 shown to be durable and resistant to solvent destruction (Datta *et al.*, 2013). Entrapment,  
141 encapsulation and cross-linking are more resistant methods to solvent interactions. In fact, it has  
142 been reported that these methods are often used to retain catalytic activity in harsh conditions  
143 (temperature and pH extremes and exposure to organic solvents) (Kourkoutas *et al.*, 2004).

#### 144 3.4 Criteria for screening organic solvents as extraction agents in downstream processes

146 Crucial criteria to consider when choosing a solvent as an extraction agent are the solubility of the  
147 target compound to be extracted, affinity towards this compound and the ease of subsequent phase  
148 separation. For instance, when extracting the product with a solvent it is important that the product  
149 is highly soluble in the solvent in order to efficiently recover most of the product from the outlet  
150 stream of the reactor (Kolář *et al.*, 2002). The ease of separation of the solvent from the aqueous  
151 phase is also important, since a complete separation reduces costs. Hence, a large density difference  
152 between the extract phase and raffinate phase (from which the components of interest have been  
153 removed) allows high capacities particularly in liquid-liquid extraction (Gu, 2000; Koch, 2015).  
154 Likewise, the higher the interfacial tension (Gu, 2000; Tzia and Liadakis, 2003), the more readily  
155 coalescence of emulsions will occur and the easier phase separation will be.

156 In some cases, the direct recovery of a product may not be possible using solvents alone and it is  
157 necessary to use a reactive liquid-liquid extraction which involves a reversible reaction between the  
158 desired chemical compound and the extractant or a host chemical species present in the extractant.  
159 Examples include the removal of carboxylic acids (acetic acid (Mahfud *et al.*, 2008), lactic acid  
160 (Wasewar *et al.*, 2003, 2002), pyruvic acid (Marti *et al.*, 2011), citric acid (Poposka *et al.*, 1998)) by  
161 amines. The extractions involve the complexation reaction of the undissociated acids and amines.  
162 The complexation reaction improves the distribution coefficient. The reaction promotes the  
163 migration of the product to the organic phase. The choice of the solvent is also important when  
164 establishing a reactive liquid-liquid extraction because it has to solvate the amine-acid complex to  
165 avoid its precipitation (Yang *et al.*, 2007).

166 Another solution, in case direct extraction is not possible, is to manipulate other properties such as  
167 modifying the pH of the output aqueous solution can be useful for separation. An excellent example  
168 of this is the downstream processing for penicillin production. After filtration of the mycelium, the  
169 pH of the broth is adjusted to pH 2-2.5 in order to convert penicillin acid carboxylate into  
170 penicillanic acid. The acidification of the broth increases the partition coefficient of penicillanic  
171 acid (Najafpour, 2007). However, penicillanic acid is unstable in aqueous solution, and this  
172 compound is recovered by an organic solvent, e.g. butyl acetate. The decision regarding the pH  
173 value to be selected should be a compromise between the partition coefficient and product stability  
174 (Wennersten, 2004) and acidification of the broth should be performed in order to minimize product  
175 degradation (Hook, 2006).

176

#### 4. Solvent selection guide for biphasic bioreaction systems

An overview of the criteria to take into account when selecting a solvent for a specific application in a process has been described in the preceding sections. In this section, a selection guide for solvents that are, or could potentially be, used in biphasic biocatalytic/fermentation reactions is described. The evaluation procedure used to rank the different solvents is similar to the CHEM21 solvent selection guide published by Prat *et al.* (Prat *et al.*, 2016).

Although there are many beneficial uses for organic solvents in bioprocesses, the use of solvents presents several environmental, health and safety challenges. When choosing a solvent for the development of a process it is important to take into account the environmental impact of the chosen solvent, and the potential safety and health risks associated with handling and using the given solvent (Clark and Tavener, 2007). Solvents having significant issues should of course be avoided, if at all possible. There are several solvent selection guides in the scientific literature. GlaxoSmithKline (GSK) (Alder *et al.*, 2016), the American Chemical Society, Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) (American Chemical Society (ACS), 2011) and the solvent guide from CHEM21 (Prat *et al.*, 2016) have presented guides with numerical rankings and dividing solvents in categories. Pfizer's (Alfonsi *et al.*, 2008) and Sanofi's (Prat *et al.*, 2013) guides present the evaluation results solely in the form of a color code for each solvent, without a numerical ranking. In addition, the solvent guide from Pfizer presents an overall summarized evaluation for all solvents, rather than divided in categories.

The survey by Prat *et al.* (Prat *et al.*, 2014) presents a summary and a comparison of the Health, Safety and Environment assessments of several solvent guides. The solvent guides considered were Astra Zeneca's, ACS GCIPR's, GSK's 2011 guide (Henderson *et al.*, 2011), Pfizer's and Sanofi's. The main purpose of this survey was to compare the evaluation criteria across the different solvent guides and compare the consistency of solvent evaluation across the various guides.

In the present article a new selection guide for solvents commonly used, or of potential use, as reaction media in biphasic biocatalysis is presented. Some of the included solvents have never been assessed in previous solvent selection guides due to their specific application in biocatalytic reaction systems. Other solvent selection guides focus strongly on solvents used in the main for synthetic organic chemistry applications (Alfonsi *et al.*, 2008; American Chemical Society (ACS), 2011; Henderson *et al.*, 2011; Prat *et al.*, 2016, 2013). An accurate and detailed comparison of all of the required properties of solvents is not an easy or exact task, since the level and quality of data available for each solvent is different. This is especially true for the comparison of older solvents that might have a large amount of data available e.g. substances fully registered under REACH (ECHA 2016), and newer solvents where very little data is available (at least available in the public domain). A key feature of the CHEM21 methodology is that it allows a high level ranking of all solvents where basic physical/safety data and the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) is known (Prat *et al.*, 2016). The solvents in the guide presented here have been classified based on the methodology developed within the CHEM21 project. CHEM21 is a collaborative project between European universities and companies and aims to develop sustainable biological and chemical alternatives to finite resources and more environmentally friendly processes. The guide presented here is targeted at process chemists and engineers charged with operating bioprocesses. In this guide we also provide some examples from the literature which document the use of the solvents in biocatalytic systems and additionally, the enzymes which have been used. Other useful data such as solubility in water, Log P and CAS number are also included. We hope these data will be useful for looking for greener solvents where similar Log P and/or water solubility values are needed for a successful bioprocess. For large scale processing, solvents which are solid close to ambient temperature can present specific logistical challenges, so solvents with

mp  $\geq 10$  °C have been marked in the table. The solvents included in the guide were chosen from a literature survey of biphasic bioreactions, or by looking for newer solvents that may have similar properties and could be good candidates for this type of transformation. Generally solvents have been chosen which have 10% or lower solubility in water as a cut off point for a water-immiscible organic solvent. In certain circumstances, solvents such as tetrahydrofuran and acetonitrile that are fully water-miscible can form biphasic mixtures with water (high aqueous solute content), but these were excluded. Data on water-miscible solvents can be found in other published guides (Alfonsi *et al.*, 2008; American Chemical Society (ACS), 2011; Henderson *et al.*, 2011; Prat *et al.*, 2016, 2013). Solvents that are common to this guide and CHEM21 used the data collated for the CHEM21 guide (Prat *et al.*, 2016). The data required to assess the new solvents were obtained from manufacturer's safety data/material safety data sheets – freely available from suppliers, and from the European Chemicals Agency, Registered Substances Data (ECHA-RS), 2016. In the case of the less common solvents and newer solvents, not all of the required data was available or found. In particular, it was difficult to find values for resistivity (the ability to accumulate a static charge). Under the methodology solvents likely to build up a static charge ( $> 10^8 \Omega.m$ ) incrementally add 1 to the safety score. For the additional solvents here, ethers and hydrocarbons were scored as resistive, and the other solvents as non-resistive. Needless to say, before using any solvents at scale, a full assessment needs to be made of all operational and safety hazards, including resistivity. Where air is used for bio oxidation and/or for transformations with living cells, appropriate care needs to be taken to avoid the formation of an explosive head space if a flammable solvent is used. Since the processes under consideration here are all biphasic, the production of aqueous waste streams containing low levels of the organic solvent needs to be considered. Some calculated data on persistence, bioaccumulation and toxicity has been included in the table. Thus the solvent selection guide includes an evaluation of persistence in the environment, bioaccumulation in food chains and toxicity to fish. The persistence, bioaccumulation and toxicity (PBT) evaluation follows the criteria established by New Chemicals Program (EPA (U.S. Environmental Protection Agency), 2012). The persistence evaluation is performed by investigating the half-life of the compound in water and air. In relation to the water criteria, if the compound's half-life is less than 2 months it is considered recommended (green). If the compound's half-life is between 2 and 6 months it is considered problematic (amber). Solvents with a half-life greater than 6 months are considered hazardous (red). Persistence in air is also evaluated by the half-life; compounds with a half-life lower or equal to 2 days are considered harmless, those with a half-life greater than 2 days are considered hazardous (EPA (U.S. Environmental Protection Agency), 2012). The bioaccumulation criterion corresponds to the bioconcentration factor of a chemical uptake from the surrounding media by an organism living in that media. If the range of bioconcentration factors is less than 1000, the solvent is considered recommended for industrial applications (green). Solvents with bioconcentration factors greater than or equal to 1000 and less than 5000 are considered to be problematic (amber). Solvents with bioconcentration factors higher than 5000 are considered hazardous and not advised to be applied in industrial applications (red) (EPA (U.S. Environmental Protection Agency), 2012). Toxicity to fish is evaluated by the concentration of the solvent which is chronically toxic to fish, chronic toxicity value (ChV). Solvents with a ChV greater than 10 mg/L and that do not present any toxic risk are considered harmless (green). ChV in the range of concentrations 0.1-10 mg/L present moderate concern and are considered problematic (amber). Solvents with ChV less than 0.1 mg/L are considered hazardous (red) (EPA (U.S. Environmental Protection Agency), 2012). Table 2 is the compilation of the assessment of the solvents which are commonly applied, or could be applied, in bioreactions as a medium. The guide contains a score for each parameter [1 (good) to 10 (bad)] and is color coded for easy reference. The guide is divided into safety, health and

environmental sections, with an overall recommendation. Scoring is based on physical parameters such as boiling point, auto ignition temperature etc. and GHS statements. Full details of the methodology are given in the CHEM21 publication (Prat *et al.*, 2016). For easy comparison in tabular form, the output is color coded. Green (recommended) indicates that the solvent can be used with few issues (given normal safe operating procedures are in place to deal with issues such as flammability, etc). Yellow (problematic) indicates that there may be some issues, but the solvent should be usable with appropriate mitigation strategies. Red - solvents labeled hazardous or highly hazardous should be replaced or avoided in developing new processes. In the overall ranking column, some solvents have a split ranking. This is due to current industrial thinking and practice that would generally move a solvent into a higher hazard band than that given by the ranking/scoring process.

For newer solvents that are not fully registered in REACH (thus potentially lacking in some data sets), the CHEM21 scoring methodology defaults to 5 (problematic/yellow). This is why solvents such as diethyl succinate and butyl butyrate rank as problematic when compared to very similar structures like ethyl, tert-butyl or isopropyl acetate, which are fully registered. When full datasets are available, these materials may become more harmonized in the guide. The REACH process is generating a lot of data on solvents and the overall picture is constantly changing. Looking into the future, before using any newer solvent, it would be advised to search for any new data or change in REACH status that could change the ranking in the table. It is worth noting that especially in the context of this guide, the methodology scores high boiling solvents (especially  $> 200\text{ }^{\circ}\text{C}$ , e.g. diethyl succinate b.p. =  $218\text{ }^{\circ}\text{C}$ ) poorly in the environmental section since these materials will be very energy intensive to purify or recover by distillation.

Lastly, the reader should note that the limits of the CHEM 21 selection algorithm define the assessment of each solvent. There are other solvent selection guides available in the literature and there are some differences in the classification of the solvents (Prat *et al.*, 2014). Moreover, the assessment limits might also change with future legislation. In line with this, we are aware that some solvents which present some toxic and flammable properties (e.g. n-butanol) currently fall into the category of “Recommended” due to the limits of the evaluation. Moreover, azeotrope formation was not considered in the selection guide, although in principle it should also be taken into account when screening for solvents due to separation problems with the recovery of the solvent or waste water treatment.

**Table 1** – Compilation of organic solvent selection guides and potential substitution solvents.

High Level Solvent Guide for Biphasic Biocatalysed Reactions												
Solvent, (CAS N°), mp if ≥ 10 °C	Solubility in water g litre <sup>-1</sup> *	Log P*	Precedent for use in biphasic bioreaction	Reference	In published guides**	Safety Score	Health Score	Environment Score	PBT profile***			Overall Ranking using CHEM21 methodology****
									P	B	T	
Alcohols												
n-Butanol (71-36-3)	63.2	0.79	Dehydrogenase	(Cremonesi <i>et al.</i> , 1973)	P, G, S, C21, RT	3	4	3				Recommended
Isobutanol (78-83-1)	70	0.79	Oxidase	(Zaks, 1988)	G, S, C21, RT	3	4	3				Recommended
n-Pentanol (71-41-0)	2.03	1.44	Decarboxylase	(Rosche <i>et al.</i> , 2004)	No	3	2	3				Recommended
n-Heptanol (111-70-6)	1.63	2.2	None found		No	1	2	5				Recommended
tert-Amyl alcohol (75-85-4)	98	0.77	Oxidase	(Zaks, 1988)	S	4	2	3				Recommended
Isoamyl alcohol (123-51-3)	21.2	1.35	None found		G, C21	3	2	3				Recommended
1-Octanol (111-87-5)	0.5	3.15	Oxygenase	(Hüsken <i>et al.</i> , 2002)	No	1	2	5				Recommended
Benzyl alcohol (100-51-6)	40	1.05	None found		G, S, C21, RT	1	2	7				Problematic
1-Dodecanol (112-53-8) mp 22 °C	0.0019	5.13	Reductase	(De Wulf and Thonart, 1989)	No	1	5	7				Problematic
1-Decanol (112-30-1)	0.021	4.5	Dehydrogenase	(Pinheiro and Cabral, 1992)	No	2	2	7				Problematic
Esters												
Ethyl acetate (141-78-6)	87.9	0.68	Dehydrogenase	(Cremonesi <i>et al.</i> , 1973)	P, G, S, C21, RT	5	3	3				Recommended
tert-Butyl acetate (540-88-5)	6.7	1.64	ω-Transaminase	(Meadows <i>et al.</i> , 2013)	G	4	1	3				Recommended
n-Butyl acetate (123-86-4)	5.3	2.3	KRED	(Ye <i>et al.</i> , 2010)	G, S, C21, RT	4	2	3				Recommended
Isobutyl acetate (110-19-0)	5.6	2.3	None found		S, C21, RT	4	2	3				Recommended

n-Propyl acetate (109-60-4)	18.7	1.27	None found		<b>G</b>	4	2	3					<b>Recommended</b>
Isopropyl acetate (108-21-4)	31.9	1.03	None found		<b>P, G, S, C21, RT</b>	4	2	3					<b>Recommended</b>
Isoamyl acetate (123-92-2)	2	2.7	None found		<b>C21</b>	3	1	5					<b>Recommended</b>
n-Butyl butyrate (109-21-7)	0.31	2.83	None found		<b>No</b>	3	5	5					<b>Problematic</b>
n-Octyl acetate (112-14-1)	0.033	3.81	P450	(Toda <i>et al.</i> , 2012)	<b>G</b>	1	5	7					<b>Problematic</b>
Diethyl succinate (123-25-1)	19	1.26	None found		<b>G, S, C21</b>	1	5	7					<b>Problematic</b>
Lauryl acetate (112-66-3)	0.00036	5.88	P450	(Garikipati <i>et al.</i> , 2009)	<b>No</b>	1	5	5					<b>Problematic</b>
Ethyl decanoate (106-33-2)	0.00041	5.71	P450	(Tan and Day, 1998)	<b>No</b>	1	5	7					<b>Problematic</b>
Ethyl oleate (111-62-6)	6x10 <sup>-7</sup>	8.51	P450	(Kuhn <i>et al.</i> , 2012)	<b>No</b>	1	5	7					<b>Problematic</b>
FAME-Fatty acid methyl esters (67762-38-3)	0.000023	>6.2	P450	(Schrewe <i>et al.</i> , 2014)	<b>No</b>	Mixture							<b>Problematic</b>
Bis n-butyl phthalate (84-74-2)	0.011	4.46	KRED	(He <i>et al.</i> , 2006)	<b>No</b>	1	9	7					<b>Hazardous</b>
bis(2-ethylhexyl) phthalate (117-81-7)	3x10 <sup>-6</sup>	7.86	P450	(Park <i>et al.</i> , 2007)	<b>No</b>	1	9	7					<b>Hazardous</b>
Tricaprylin (538-23-8)	1.5x10 <sup>-8</sup>	9.2	Plant cell culture	(Dutta, 1994)	<b>No</b>	1	5	7					<b>Problematic</b>
<b>Ketones</b>													
Methyl Isobutyl ketone (MIBK) (108-10-1)	14.1	1.9	α-Galactosidase	(Bennett <i>et al.</i> , 1992)	<b>S,G,C21, RT</b>	4	2	3					<b>Recommended</b>
Cyclohexanone (108-94-1)	90	0.86	Imidase	(Ogawa <i>et al.</i> , 2000)	<b>G, S, C21, RT</b>	3	3	5				<b>R</b>	<b>P</b>
2-Octanone (111-13-7)	0.9	2.5	KRED	(Kohlmann <i>et al.</i> , 2011)	<b>No</b>	3	5	5					<b>Problematic</b>
2-Undecanone (112-12-9) mp 15 °C	0.04	4.1	Oxidation	(Collins and Daugulis, 1997)	<b>No</b>	1	5	7					<b>Problematic</b>
<b>Ethers</b>													



Dimethyl ether <sup>†</sup> (115-10-6)	335	0.07	KRED	(Lu <i>et al.</i> , 2004)	<b>G</b>	9	2	7				H	HH
Diethyl ether (60-29-7)	43.1	1.05	Dehydrogenase	(Cremonesi <i>et al.</i> , 1973)	<b>P, G, S, C21, RT</b>	10	3	7				H	HH
Diisopropyl ether (108-20-3)	3.1	1.52	Enoate reductase	(Hall <i>et al.</i> , 2012)	<b>P, G, S, C21, RT</b>	9	3	5				Hazardous	
Dibutyl ether (142-96-1)	0.11	3.35	ω-Transaminase	(Meadows <i>et al.</i> , 2013)	<b>S</b>	5	2	5				Problematic	
2-Methyltetrahydrofuran (96-47-9)	140	1.1	Benzaldehyde lyase	(Shanmuganathan <i>et al.</i> , 2011)	<b>P, G, S, C21, RT</b>	6	3	3				R	P
Cyclopentyl methyl ether (CPME) (5614-37-9)	3.1	1.59	Benzaldehyde lyase	(Wiedner <i>et al.</i> , 2015)	<b>G, S, C21, RT</b>	7	2	5				Problematic	
tert-Butyl methyl ether (TBME) (1634-04-4)	41.9	1.23	Hydroxy nitrile Lyase	(Wiedner <i>et al.</i> , 2015)	<b>P, G, S, C21, RT</b>	8	3	5				Hazardous	
Ethyl tert-butyl ether (ETBE) (637-92-3)	2.37	1.48	None found		<b>G, S, C21</b>	7	3	3				Problematic	
tert-Amyl methyl ether (TAME) (994-05-8)	10.7	1.55	None found		<b>G, C21</b>	6	2	3				Recommended	
Diisoamyl ether (544-01-4)	0.028	5.08	Dehydrogenase	(Hocknull and Lilly, 1990)	<b>No</b>	4	2	7				Problematic	
Anisole (100-66-3)	1.71	2.11	Lipase	(Wells, 2010)	<b>G, S, C21, RT</b>	4	1	5				P	R
<b>Halogenated</b>													
Dichloromethane (DCM) (75-09-2)	13.2	1.25	Dehydrogenase	(Cremonesi <i>et al.</i> , 1973)	<b>P, G, S, C21, RT</b>	1	7	7				Hazardous	
Chloroform (67-66-3)	8.7	1.97	Protease	(Ogino <i>et al.</i> , 1995)	<b>P, G, S, C21, RT</b>	2	7	5				P	HH
Carbon tetrachloride (56-23-5)	0.65	2.64	Oxidase	(Liu <i>et al.</i> , 1996)	<b>P, G, S, C21, RT</b>	2	7	10				H	HH
1,2-Dichloroethane (107-06-2)	7.9	1.45	None found		<b>P, G, S, C21, RT</b>	4	10	3				H	HH
Chlorobenzene (108-90-7)	0.21	2.98	Dehydrogenase	(Cremonesi, 1975)	<b>G, S, C21, RT</b>	3	2	7				Problematic	
Methoxyperfluorobutane (163702-07-6)	0.01	3.93	Nitrile hydratase	(Zhu <i>et al.</i> , 2015)	<b>No</b>	3	6	5				Problematic	
Benzotrifluoride	0.21	3.01	None found		<b>G, RT</b>	5	5	7				Problematic	

(98-08-8)														
Aromatic hydrocarbons														
Benzene (71-43-2)	1.78	2.1	KRED	(Shi <i>et al.</i> , 2008)	P, G, S, C21, RT	6	10	3					H	HH
Toluene (108-88-3)	0.52	2.73	Nitrile hydratase	(Cull <i>et al.</i> , 2001)	P, G, S, C21	5	6	3					Problematic	
Xylene (1330-20-7)	0.16	3.15	Oxidase	(Aono <i>et al.</i> , 1994)	P, G, S, C21, RT	4	2	5					Problematic	
p-Cymene (99-87-6)	0.03	4.1	Lipase	(Paggiola <i>et al.</i> , 2014)	G, S, C21	4	5	5					Problematic	
Tetralin (119-64-2)	0.045	3.78	Reductase	(Ferrante <i>et al.</i> , 1995)	S	3	6	7					Problematic	
Cumene (98-82-8)	0.05	3.55	None found		S, G	5	2	7					Problematic	
Aliphatic hydrocarbons														
n-Pentane (109-66-0)	0.039	3.45	None found		P, G, S, C21	8	3	7					Hazardous	
n-Hexane (110-54-3)	0.01	4	KRED	(de Gonzalo <i>et al.</i> , 2007)	P, G, S, C21, RT	8	7	7					Hazardous	
n-Heptane (142-82-5)	0.0024	4.5	Dehalogenase	(Zou, 2014)	P, G, S, C21, RT	6	2	7					Problematic	
n-Octane (111-65-9)	0.0007	5.15	Nitroreductase	(Meyer <i>et al.</i> , 2006)	S, C21	5	2	7					Problematic	
Isooctane (540-84-1)	0.0022	4.08	Lipoxygenase	(Kermasha <i>et al.</i> , 2002)	G, RT	6	2	7					Problematic	
Cyclohexane (110-82-7)	0.052	3.44	Esterase	(Lee, 1997)	P, G, S, C21, RT	6	3	7					Problematic	
Methylcyclohexane (108-87-2)	0.014	3.88	None found		P, G, S, C21, RT	6	2	7					Problematic	
Petroleum ether 60/80 (101316-46-5)	As n-hexane	As n-hexane	KRED	(Pathan <i>et al.</i> , 2012)	G	Mixture						P	H	
Paraffin oil (8012-95-1)	Insoluble	>4	Oxidase	(Oda <i>et al.</i> , 1996)	No	Mixture						P	H	
Decane (124-18-5)	0.000083	5.86	Expandase	(Gao and Demain, 2001)	No	4	2	5					Problematic	
Dodecane (112-40-3)	0.000005	6.98	KRED	(Huang <i>et al.</i> , 2005)	No	2	2	7					Problematic	
Tetradecane	2.8x10 <sup>-7</sup>	7.2	Dioxygenase	(Collins <i>et al.</i> ,	No	2	2	7					Problematic	

(629-59-4)				1995)								
Hexadecane (544-76-3) mp 18 °C	0.000001	8.20	P450	(Furuhashi, 1986)	No	2	2	7				Problematic
D-Limonene (5989-27-5)	0.006	4.4	Hydratase	(Savithiry <i>et al.</i> , 1997)	S, C21	4	2	7				Problematic
Turpentine (8006-64-2)	0.002 to 0.35	3 to 6	None found		S, C21	4	2	7				Problematic

\* Data from ECHA database [(ECHA-RS), 2016], literature values (sourced from the Reaxys database, Chemspider) or calculated. Values between 20 and 30 °C.

\*\* Solvent listed in other guides P=Pfizer (Alfonsi *et al.*, 2008), G= GSK (Alder *et al.*, 2016), S= Sanofi (Prat *et al.*, 2013), C21= CHEM21 (Prat *et al.*, 2016), RT= ACS GCI Pharmaceutical Roundtable (ACS 2011).

Grey shading indicates scoring is not appropriate due to mixtures, or values cannot be calculated for PBT profiler (Environmental Health Analysis Center, 2012)

\*\*\* Calculated environmental fate <http://www.pbtprofiler.net/>

P = Persistence

B = Bioaccumulation

T = Toxicity to fish

\*\*\*\* Recommendation as an output from the CHEM21 solvent selection methodology (Prat *et al.*, 2016). Where a cell is split, the first column represents the output from the tool. However, for certain solvents, a second column has been added to reflect current industrial practice and thinking.

† solvent used under pressure, the boiling point of dimethyl ether -24 °C at atmospheric pressure.

## 319 5. Concluding remarks and future perspectives

320 This article summarizes water-immiscible solvent applications in bioprocesses and enumerates the  
321 different criteria to take into account in order to select a solvent. The criteria have been compiled  
322 and organized in a screening procedure which helps to narrow down the number of potentially  
323 feasible solvents to be tested experimentally during early stage process development, and to help  
324 guide chemists and engineers towards solvents with the best EHS profiles. The most important  
325 properties that are necessary to consider when screening organic solvents for a process are related to  
326 their environment, health and safety impact, recoverability and stability and their application in the  
327 process, as a reaction medium or applied to downstream processing.

328 Unfortunately, an ideal solvent is not always available from the shortlist of solvent options, and it is  
329 not always possible to fulfill all of the requirements. For example solvents with high Log P values  
330 are favored for two-liquid phase systems with free, immobilized biocatalysts or whole cells,  
331 whereas these are the very solvents which tend to persist in the environment and score poorly in the  
332 environmental assessment of the solvent guide. More lipophilic solvents also tend to have higher  
333 resistivity and consequently a higher safety score. Therefore, when making the final choice it is  
334 necessary to take a decision about the importance of the evaluation categories and to set strategies  
335 to overcome the constraints of the unfulfilled requirements. These strategies should still establish a  
336 safe and environmentally friendly process with reasonable acceptable costs.

337 Moreover, sometimes there are also process challenges to overcome such as deactivation of the  
338 biocatalyst in the presence of an organic solvent. This can often be overcome by using an indirect  
339 solvent contact process. In fact, it is also possible to avoid the contact of the biocatalyst with the  
340 solvent by making the extraction outside of the reactor without recirculation of the aqueous phase to  
341 the reactor – Figure 1.

342 The selection of solvents for application in industrial processes has been changing over the past two  
343 decades. In fact, today there is a tendency both in industry and in research to choose a solvent  
344 taking greater consideration of the environmental impact and also an impact on health, safety and  
345 costs aspects. As an example GlaxoSmithKline Pharmaceuticals' most frequently used solvents list  
346 has changed towards greener solvents. Solvents such as toluene, dichloromethane and  
347 tetrahydrofuran, which were applied greatly in industry in the 90's, are presently being replaced.  
348 The three top ranked solvents for industrial application were 2-propanol, ethyl acetate and  
349 methanol. The list of the 10 top ranked solvents includes also ethanol, n-heptane, tetrahydrofuran,  
350 toluene, dichloromethane, acetic acid and acetonitrile (Constable *et al.*, 2007). Moreover, a survey  
351 of solvent usage in development of processes revealed that although there is some room for  
352 improvement on substituting solvents of concern, there is already some reduction of chloroform and  
353 n-hexane applications. Additionally, this investigation shows that the usage of dipolar aprotic  
354 solvents at larger scale (>100 kg scale) is much smaller than in processes at smaller scale (Ashcroft  
355 *et al.*, 2015). Another factor driving industry towards more benign solvents is legislation, especially  
356 Registration and Evaluation of Chemicals (REACH) in the EU which seeks to limit and eventually  
357 remove from use substances with carcinogenic, reprotoxic and mutagenic properties, as well as  
358 materials with a high environmental impact (European Chemicals Agency (ECHA), 2016).

359 The scientific community has focused research to find greener solvents for bioprocesses and these  
360 efforts are centered on the application of ionic liquids, deep eutectic solvents and supercritical  
361 carbon dioxide (Jessop, 2011). Ionic liquids have been extensively studied by the scientific  
362 community as possible reaction media for biocatalysis. Ionic liquids are mixtures of cations and  
363 anions which do not pack well and therefore, these mixtures are in liquid phase at room  
364 temperature. Several enzymes have been tested having ionic liquids or a mixture of ionic liquid and  
365 water as reaction media. From the scientific literature, it is possible to conclude that in ionic liquids

366 several enzymes present good stereoselectivity, reaction yield, activity and stability (Lou *et al.*,  
 367 2005; Lozano *et al.*, 2001). For example, Lozano and coworkers have studied  $\alpha$ -chymotrypsin and  
 368 verified an increase of its half-life and the conversion of the substrate when compared to 1-propanol  
 369 (Lozano *et al.*, 2001). The implementation of ionic liquids in industrial processes will require more  
 370 information regarding their toxicity, ecotoxicity and their life cycle impact. Moreover the  
 371 ecotoxicity of the ionic liquid seems to be related to the branching of the alkyl chain and to  
 372 hydrophobicity of the cation (Docherty and Kulpa, Jr., 2005). Some of the ionic liquids have EC<sub>50</sub>  
 373 (acute toxicity value) values much lower than for example toluene, which means they are more  
 374 ecotoxic. Another aspect to consider when evaluating the environmental impact of ionic liquids is  
 375 the environmental impact of their synthesis. The synthesis of an ionic liquid sometimes requires the  
 376 use of harmful organic solvents (Deetlefs and Seddon, 2010; Zhang *et al.*, 2008). There have also  
 377 been efforts to decrease the toxicity of ionic liquids. In fact the third generation of ionic liquids has  
 378 been considered cheaper, sustainable, non-toxic and biodegradable (Domínguez de María and  
 379 Maugeri, 2011; Fukaya *et al.*, 2007).

380 Supercritical carbon dioxide (scCO<sub>2</sub>) is also considered a sustainable solvent since it is non-  
 381 flammable, has low toxicity, is broadly inert limiting unwanted reactions, and is present in  
 382 abundance as a by-product of industrial processes like fermentation and thermal cracking. Although  
 383 scCO<sub>2</sub> presents several advantages at safety and process level, it has also some associated  
 384 disadvantages. Some organic substrates have poor solubility in scCO<sub>2</sub>, requiring the use of a co-  
 385 solvent. A process which uses supercritical carbon dioxide requires high pressure equipment and  
 386 therefore it is necessary to consider carefully the safety aspects. Furthermore, another constraint of  
 387 the application of scCO<sub>2</sub> in a process is the cost of operation and equipment capital cost which is  
 388 much higher compared to a standard organic solvent since the process has to operate at high  
 389 pressure (Beckman, 2004). Concerning application in bioprocesses, studies have demonstrated that  
 390 scCO<sub>2</sub> can improve reaction rates and control reaction selectivity by pressure. Many enzymes have  
 391 been demonstrated to have a high performance in scCO<sub>2</sub> compared to organic solvents. Examples  
 392 include hydrolases, oxygenases and dehydrogenases, and have been reviewed by Wimmer and  
 393 Zarevúcka, 2010. In addition, lipases seem to have been extensively studied and reported in the  
 394 scientific literature (Khosravi-Darani and Mozafari, 2009). However, the enzyme is not always  
 395 stable in a biphasic CO<sub>2</sub>/H<sub>2</sub>O system due to the dissolution of CO<sub>2</sub> in water which causes the  
 396 formation of H<sub>2</sub>CO<sub>3</sub>. Consequently, pH will decrease (2.85) and the enzyme can be deactivated. In  
 397 addition, carbon dioxide is a Lewis acid and reacts with strong bases and nucleophiles (Beckman,  
 398 2004). Therefore, it is necessary to take this fact into account when considering the application of  
 399 scCO<sub>2</sub> in processes in which these compounds are substrates or products.

400 The solvent for a process can be chosen from several categories of solvents: water, organic solvents,  
 401 ionic liquids and supercritical fluids. Jessop has consulted top academic experts in green solvents  
 402 about which solvents they would choose for industrial application, and the choice fell on water,  
 403 supercritical carbon dioxide and carefully-selected organic solvents (Jessop, 2011).

404 In conclusion, the choice of a solvent for a bioprocess should comprise a balance between the  
 405 effects on the environment, effects on human health, safety hazards, biocatalyst/microorganism  
 406 activity, solubility and selectivity of substrates and/or products and recovery. This balance is  
 407 important because it is not always possible to find a solvent which fully covers all these criteria.  
 408 Problems regarding the impact of a solvent on Environment, Health and Safety are increasingly  
 409 being taken into account in process development when considering the application of a solvent as a  
 410 reaction medium or as part of downstream processing in new processes. Moreover, in recent years,  
 411 there has been more focus to substitute the hazardous solvents in already running processes.

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421

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